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Estrogens promote growth of breast and endometrial cancers. Selective estrogen receptor modulators (SERMs) block the activity of estrogen in selective tissues and control the growth of these cancers while avoiding many unwanted side effects associated with the use of antiestrogens. The goal of our work is to identify mechanisms regulating the activity of SERMs and to develop strategies for the identification of novel SERMs.

SERMs exert their activities by binding to the two estrogen receptors, ERα and ERβ. While the characterization of ligand-bound ERs by X-ray crystallography gave many insights into ligand binding, they do not provide a coherent explanation for the tissue- and receptor isotype-specific activities displayed by many ligands. Our results indicate that the F-domain, a C-terminal extension of the ligand binding domain (LBD) regulates ligand and coregulator binding in a receptor-specific manner. Moreover, SERMs might differ from pure agonists or antagonists in terms of the dynamics rather than the nature of the ligand-induced structural changes. We have constructed a series of fluorescent ER LBD derivatives that will not only give novel insights into the actions of SERMs but also provide a powerful strategy for the identification of novel SERMs for breast cancer therapy.

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Introduction

Estrogens promote the growth of particular tissues, and are involved in the cause of breast and endometrial cancers. Antiestrogens block the activity of estrogens and play important roles in the treatment of these cancers. However, they have many unwanted side effects such as increased risk for osteoporosis and heart diseases. By blocking the activity of estrogens in some but not in all tissues, selective estrogen receptor modulators (SERMs) provide a powerful alternative to antiestrogens. The goal of our work is to identify the mechanisms that control the activity of SERMs and to develop efficient high-throughput strategies for their identification.

Like natural estrogens, SERMs exert their activities by binding to the two estrogen receptors, ERα and ERβ, which are ligand-regulated transcription factors. The current hypothesis is that the cell-specific activity of SERMs is caused by cellular differences in the repertoir of coactivators and corepressors that recognize particular, SERM-induced receptor conformations.

Upon ligand binding, the position of α–helix 12 (H12) in the ligand-binding domain (LBD) of ERα and ERβ changes in a ligand-specific manner and regulates the interaction of these receptors with cellular corepressors and coactivators. The **first specific aim** of our proposal is to monitor the location and dynamic of α-helix 12 of ER in the absence and presence of various estrogens and develop a high throughput screen for the identification of new potential SERMs.

In addition to developing strategies to monitor ligand-induced changes in the ER LBD, we also wanted to identify structural features of ER involved in the regulation of these ligand-dependent conformational changes, such as the F-domain or the formation of homo- or hetrodimers.

The F-domain, which is the focus of our **second specific aim**, extends the C-terminus of H12. The F-domains of ERα and ERβ are very diverse in sequence. Mutations in the F-domain have been identified that enable steroid receptors to activate transcription in the presence of antagonists (Montano et al., 1996; Nichols et al., 1998) indicating that these domains play important roles in the ligand interpretation of steroid receptors. Unfortunately, since the ER F-domain seems to impede crystallization, all available ER structures miss this important domain. The recently solved structures of the progesterone and glucocorticoid receptors, which include this domain, demonstrate that in the presence of agonists the F-domain is linked to the ligand binding domain via a β-strand (Williams and Sigler, 1998; Bledsoe et al., 2002). In this specific aim we will investigate whether the F-domain restricts the mobility of H12 and modulates the ligand-induced relocation of this helix. Moreover, since the the F-domain is located close to the coactivator and corepressor binding sites in the ER LBD, we will monitor the influence of this domain on the binding of ER to hormone and coregulators.

In our **third specific aim** we proposed to probe for H12-dependent structural changes in ERα and ERβ homo- and heterodimers using fluorescence-labeled ERs. Recent results indicate that hormone binding of one dimerization partner is sufficient to induce hormone-like structural changes into the other dimerization partner (Tremblay et al., 1999). To investigate the structural basis of this activation, we planned to study the location and dynamics of H12 of a fluorescence labeled ERα mutant that cannot bind hormone itself, upon interaction with hormone-bound, but unlabeled ERβ. However, due to the difficulties we had in finding a valuable strategy to introduce fluorescent labels into ER and with respect to our difficult personnel situation (see comment at the end of this report), during the period of this grant we have focused entirely on specific aims 1 and 2.

Research Accomplishments

Specific Aim 1

Monitor the location and dynamic of H12 of ER in the absence and presence of various estrogens and develop a high throughput screen for the identification of new potential SERMs

The biological activity of an ER ligand is determined by the cofactors that are recruited by the ligand-bound receptor. Since the recruitment of these cofactors by ER is regulated by ligand-induced structural changes in the receptor, monitoring these structural changes by fluorescence anisotropy, fluorescence resonance energy transfer or pyrene excimer fluorescence are powerful strategies to characterize the potential actions of ligands and to identify ligands with new activity profiles.

In aim 2 we demonstrate that ERα and ERβ have different biochemical features and mechanisms to regulate the recruitment of coactivators. Although it will be ultimately very interesting to compare the structural changes in ERα and ERβ upon binding to particular ligands, we decided to initially focus on ERβ, mainly because this receptor is more soluble. Moreover, since the F-domain of ERβ does not contribute to ligand- and coactivator-binding, the fluorescence studies can be performed with a C-terminally truncated form of the ERβ LBD for which structural information is available.

To introduce the fluorophore two strategies were tested:

(A) The incorporation of fluorophores at specific sites using stop-codon suppression. Using this strategy we would be able to label up to two specific sites (a donor and an acceptor) within the ER ligand binding domain, which would allow us to monitor ligand-dependent changes in ER by fluorescence energy transfer (FRET). However, this system has the disadvantage that it is experimentally very difficult.

(B) A protein splicing approach. In this system, the yeast protein intein is genetically fused to the C-terminal end of an ER LBD fragment. Triggered by free sulphorhydryl groups, intein catalyzes a transesteration reaction that can be used to link an *in vitro* synthesized and fluorescence-labeled peptide or a fluorophore-linked cysteine to the C-terminal end of this ER fragment. This approach allows the introduction of a fluorophore into specific sites of either the F-domain or H12. Ligand-dependent changes in the ER LBD could then be monitored by steady state or time resolved fluorescence anisotropy.

Our experimental approach for this aim has been:

1a) To select and characterize residues within the ER LBD that are suitable for the introduction of a fluorophore;
1b-d) To test various strategies to introduce the fluorophore;
1e) To express and purify fluorescence-labeled ER;
1f) To analyze ER bound to agonists and SERMs by fluorescence spectroscopy;
1g) To develop a high throughput strategy to screen chemical libraries for potential SERMs by monitoring the fluorescence properties of ligand-bound, fluorescence-labeled ER

Aim 1a: Selection and characterization of sites for the introduction of fluorophores

Criteria for the selection of sites - Ideally, incorporated fluorophores should be solvent-accessible and should not interfere with protein folding, stability, and ligand binding.

Moreover donors and acceptors should be located such that the fluorescent properties of donors and acceptors are sensitive to the structural changes induced by the ligand.

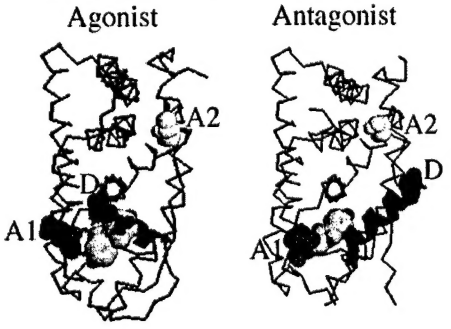


Fig. 1 Possible sites for the incorporation of fluorescence donors (D) and acceptors (A). The agonist structure is based on ERα LBD(DES), the antagonist structure ERβ LBD(Gen). H12 is shown in red, the ligands (DES, Gen) are shown in space-fill representation. In ERβ possible sites for the fluorescence donor are N496, A497, H498 (shown), possible sites for A1 and A2 are L477, T323, respectively. In the presence of an agonist the fluorescence donor (D) in H12 is very close (8 Å) to the acceptor group A1 and 20 Å apart from A2. In the presence of an antagonist, D moves closer to the acceptor group A2 (12 Å). In this situation the distance between D and A1 is 24 Å. In contrast to the position of D, the location of A1 and A2 is not ligand-dependent. The positions of D, A1 and A2 seems not to be affected by dimerization of ER.

Based on available structural information, we selected the following sites for the introduction of the fluorophores: Potential donor sites - N496, A497, H498; potential acceptor sites - L477 (A1-Agonist monitor), T323 (A2-Antagonist monitor).

Mutational analysis of potential donor sites - To determine whether replacement of the selected sites by a bulky, hydrophobic residue would interfere with protein folding, stability, and ligand binding, we replaced the selected donor sites by tryptophan using site-directed mutagenesis, expressed the mutant proteins in context of ERβ LBD±F-domain in *E. coli*, purified the protein and measured the affinity of these proteins for estradiol and 4OH-tamoxifen. In the presence of the F-domain the mutants N496W, A497W and H498W bind estradiol and 4OH-tamoxifen with similar affinity than wild type ERβ (1.4 ± 0.5 nM, 30.1 ± 5.3 nM, respectively). However in the absence of the F-domain the affinity of N496W and A497W for estradiol increased by a factor two, whereas the affinity of ERβ and H498W for estradiol remained more or less unchanged. Thus, H498 seems to be the best candidate residue for the introduction of the fluorescence donor. The affinity of N496W, A497W and H498W for 4OH-tamoxifen was not affected by the presence or absence of the F-domain.

Aim 1b-d: Analysis of various strategies to introduce the fluorescence labels

A) Nonsense codon suppression strategy

Evaluation of in vitro expression systems - In the original proposal, we plan to incorporate the fluorophores into the ER LBD using a nonsense-codon suppression strategy (Fig. 2). This strategy requires a very efficient protein *in vitro* translation system. To identify the optimal system for our purpose, we compared the overall yield and hormone-binding activity of ERβ LBD expressed i*n vitro* using bacterial, wheat germ or rabbit reticulocyte lysates (Aim 1b). We found that the overall protein yield was highest in the bacterial expression system, however the yield of hormone-binding competent receptor was significantly higher for proteins expressed in reticulocyte lysate (*E. coli* - yield: 10-50 µg protein/ ml lysate, 50-60% hormone binding competent; rabbit reticulocyte lysate - yield 0.5-5 µg protein/ ml lysate, up to 90% hormone binding competent). Based on these results, we decided to express ER in a rabbit reticulocyte *in vitro* translation system. We attribute the higher hormone binding competence of ER expressed in reticulocyte lysate to the presence of the Hsp90 chaperone complex, which is known to stabilize the hormone-binding-competent conformation of steroid receptors.

Nonsense-codon suppression - Introduction of fluorophores by nonsense-codon suppression is a difficult experimental strategy that requires careful planning of every experimental step. With the help of Dr. Peggy Saks, an expert in tRNA evolution and protein synthesis (Saks et al., 1996), we constructed nonsense-codon suppressor tRNAs that can be charged *in vitro* with cysteine but are not recognized by aminoacyl synthetases present in the lysate (Aim 1c). We produced these tRNAs using an *in vitro* expression system, and charged them with cysteine. In a second step the tRNA-bound cysteines were labeled with the fluorophore. In *in vitro* expression trials we found that these suppressor tRNAs drastically reduced the yield of *in vitro* expressed ER LBD, even if they were charged with cysteine without the fluorescence label. Moroever, although we consulted with Molecular Probes (Eugene, OR), we had difficulties to identify fluorophores that did not inhibit recognition of the loaded tRNAs by the ribosome and whose Stoke's radii were sufficiently different to distinguish A1-D and A2-D interactions of the fluorophores.

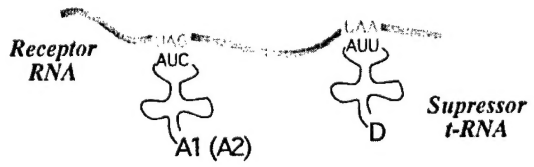


Fig. 2 Nonsense-codon suppression
Nonsense-codon suppressor tRNAs are coupled *in vitro* with cysteine residues linked to fluorophores (A1, A2, or D). *In vitro* translation of mRNAs containing the corresponding nonsense codons in the presence of these tRNAs yields proteins that contain fluorescence labels at positions determined by these nonsense codons.

B) Protein splicing approach

As an alternative approach to incorporate a fluorophore into H12 we tried protein splicing. For this strategy we synthesized ERβ H12 containing a fluorescein labeled cysteine residue at position 498 and coupled this helix to a C-terminally truncated ERβ LBD using an intein-catalyzed protein labeling strategy as outlined in Fig. 3 (Aim 1d). In test trials using the

progesterone receptor (PR) LBD and an unlabeled H12, this strategy worked great (see specific aim 2). However, for both, ER and PR, the fluorophore decreased the solubility of H12 to the point where the ligation reaction became very inefficient.

Last year Kallenberger et al. (2003) reported a study that contained a possible solution to our problems. This study investigates the dynamic properties of H12 in the peroxisome proliferator-activated receptor γ (PPARγ) by monitoring the mobility of a fluorophore coupled to the C-terminus of H12 using fluorescence anisotropy. These authors also used an intein-dependent protein splicing system. However, instead of coupling a fluorescence-labeled peptide, they coupled cysteine-fluorescein to the C terminus of the PPARγ LBD. This approach circumvents the problems caused by the low yield and solubility of fluorophore-containing H12 peptide.

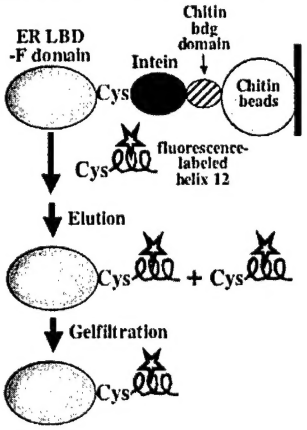


Fig. 3 Fluorescence-labeling of ER by protein splicing
This strategy requires a C-terminally truncated fragment of the ERβ LBD that lacks the F-domain and H12. This ERβ fragment is linked via a cysteine residues to a modified splicing domain of the yeast protein intein. This fusion protein can be purified by binding to chitin beads via a chitin binding domain (CBD) that has been engineered at the C-terminus of the intein domain. In the presence of free thiols the linkage between the ER domain and intein is cleaved. In our case, the free thiol is at the N-terminal end of a synthetically synthesized and fluorescence-labeled H12. Hence breaking the linkage between the ER LBD domain and intein will result in linking the labeled helix at the C-terminus of the ER LBD. Upon elution of the labeled ER LBD from the chitin column, free H12 peptides are removed by gel filtration.

Selection of C-terminal cleavage sites - In the last three years structures of ERβ bound to ICI 164,384, Raloxifene, Genistein, (R,R)-5,11-cis-Diethyl-5,6,11,12-Tetrahydrochrysene-2,8-Diol (DTCD) and Triazine have been published (Pike et al., 2001; Pike et al, 1999; Shiau et al., 2002; Henke et al., 2002). Unfortunately, in the structure of ERβ bound to the antiestrogen ICI 164,384 no electron density has been identified for H12. In the remaining four structures the extension and position of helix 12 vary in a ligand-dependent manner (Fig. 4). Because our goal is to monitor the ligand-dependent movement of H12, the fluorophore has to be introduced as closly as possible to the C-terminal end of this helix. Based on the available structures we decided to cleave the C-terminus of ERβ at the following positions: A497, L500, and R501. Because ERβ-F constructs used in specific aim 2 extend to cysteine 503, we also constructed an ERβ version that terminates at C503. Based on available structural information, these residues are solvent exposed and hence accessible for the splicing reaction. In specific aim 2 we show that ERβ cleaved at position 503 binds 17ß estradiol, 4OH-tamoxifen, GRIP1 and NCoR with similar affinity than wild type ERβ.

Construction, expression and purification of ERβ LBD: intein fusion proteins - Since linkage efficiency and ligand-independent movement of H12 might be influenced by the sequence of the ER LBD: intein junction, we constructed a series of C-terminal ERβ: intein fusion proteins comprising the ERβ LBD fragments 258-497, 258-500, 258-501 and 258-503. The junction sequences or labeled C-termini of the corresponding ERβ LBD proteins are shown in (Tab. 1).

ERβ construct	ERβ LBD: intein fusion	Fluorescent labeled ERβ LBD
ERβ 258-497(G)	ERβ - DLLLEMLNA**GC** - **Intein**	ERβ - DLLLEMLNA**GC***
ERβ 258-500 (G)	ERβ - DLLLEMLNAHVL**GC** - **Intein**	ERβ - DLLLEMLNAHVL**GC***
ERβ 258-503 (G)	ERβ - DLLLEMLNAHVLRGC - **Intein**	ERβ - DLLLEMLNAHVLRGC*
ERβ 258-501 (P)	ERβ - DLLLEMLNAHVLRPC - **Intein**	ERβ - DLLLEMLNAHVLR**PC***
ERβ 258-501	ERβ - DLLLEMLNAHVLRC - **Intein**	ERβ - DLLLEMLNAHVLR**C***

Tab. 1 ERβ: intein junction sequences and labeled ERβ C-termini of the ERβ variants 258-497, 258-500, 258-501 and 258-503. Heterologous sequences not present in ERβ are in bold. "*" symbolizes the fluorescence label.

Due to the construction of the pTYB2 expression vectors, a glycine residue precedes the spliced cysteine residue in the protein: intein linkage. We are concerned that this design will increase the H12 independent rotation of the fluorophore, which could impair the detection of the H12 movement. Therefore, we mutated the cloning site of pTYB2 to allow the construction of ER: intein fusions where a proline residue precedes the fluorophore linkage residue (ERβ 258-501 (P) variants) (Tab. 1).

Fluorophore selection and synthesis - We used fluorescein as fluorescence label because (1) fluorescein has been already successfully used to monitor the mobility of H12 (Kallenberger et al., 2003), and (2) fluorescein is negatively charged. As shown in Fig. 4, the conformational changes of H12 induced by different ligands bring the C-terminus of H12 into environments that differ in their charge distribution. Hence, a charged fluorophore has the potential to be sensitive to these changes and might facilitate the detection of differences in the location or mobility of H12.

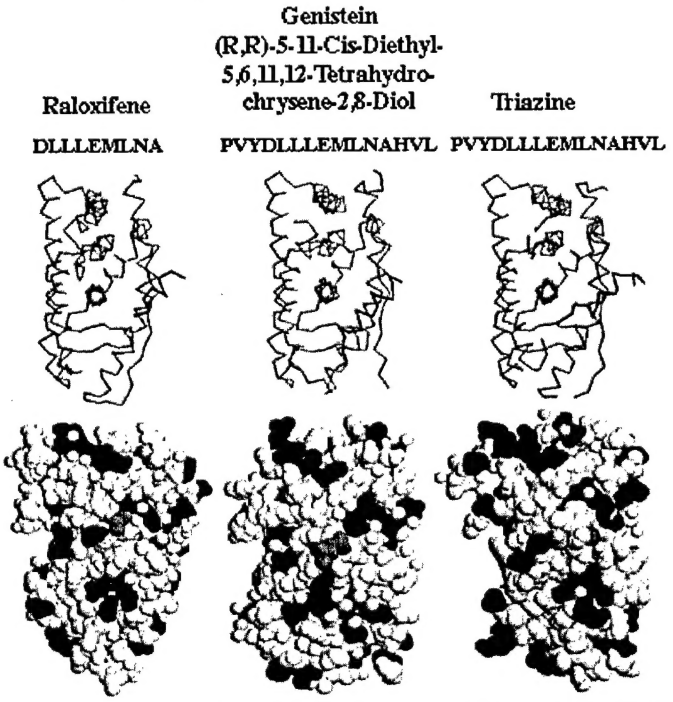


Fig. 4 Structures of ERβ LBD bound to raloxifene, genistein or DTCD, and Triazine (Pike et al, 1999; Shiau et al., 2002; Henke et al., 2002). In the backbone presentations H12 is labeled black. For each of these structures, the sequence of H12 is shown above the picture. The green residue in the space-filled representations of the ERβ LBD markes the C-terminus of H12. Solvent exposed positively charged residues are shown in blue, negatively charged residues are red.

We had made arrangements with Molecular Probes (Eugene) to obtain the cysteine-fluorescein used by Kallenberger et al. (2003). However, during the course of this work Molecular Probes was purchased by Invitrogen, which decided to reconstruct the infrastructure of Molecular Probes and put most of their collaborations on hold. Due to these circumstances thus far we have only obtained a small quantity of the substance. Initially, we thought that linking fluorescein to 2-mercapto-ethylamine would provide an alternative strategy to induce the cleavage reaction. Unfortunately, this strategy won't work since we also need an aminoacyl group to initiate splicing. We are presently working on alternative arrangements such as collaborations with chemists in our department.

Aim 1e: Expression and purification of fluorescence-labeled ER

During the last year we expressed the ERβ 258-497(G), ERβ 258-500(G), ERβ 258-503(G), ERβ 258-501(P), ERβ 258-501 intein fusion proteins in *E. coli* and purified them by binding to chitin beads using a chitin binding domain, which has been engineered at the C-terminus of the intein domain. These intein fusion proteins are relatively large compared to the average size of *E. coli* proteins, which reduces the expression of these proteins to some extent. Most of our intein fusion proteins were expressed in yields between 0.2 - 1.0 mg protein per liter culture and are about 30% soluble (Fig. 5, lane A). To evaluate the efficiency of our purification protocol, we cleaved the ER domain of chitin bead-bound ER: intein fusion proteins using DTT. As shown on the example of ERβ 258-503(G) (Fig. 5, lane B), the eluted proteins are more than 90% pure. One major contamination is the unlabeled ER: intein protein released from the

column. We next tested whether these ER: intein proteins could be cleaved using cysteine-fluorescein and whether the resulting proteins are fluorescent. Since at present we only have a small amount of cysteine-fluorescein, these experiments were performed in small scale. As shown in Fig. 5, lane C, cysteine-fluorescein cleaved the ER LBD fragment. To remove unbound fluorophores we purified the eluted protein by gelfiltration. Fluorescence analysis confirmed that the resulting protein contains fluorescein. Overnight dialysis did not change the fluorescence confirming that the fluorescence comes form the protein and not from residual cysteine-fluorescein. Thus, using this strategy we will be able to generate ERβ LBD variants whose H12 is fluorescence-labeled. The small scale protein preparations we have done thus far might be sufficient to decide which of the five constructs are best for the planned anisotropy studies.

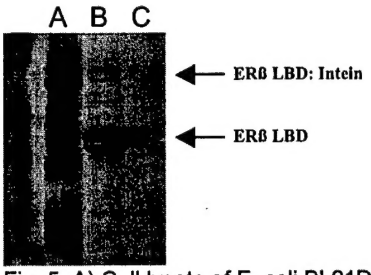


Fig. 5 A) Cell lysate of E. coli BL21DE3 expressing ERβ 258-503(G): intein. B) Chitin bead purified and DTT cleaved ERβ 258-503(G). C) Chitin bead purified and cysteine-fluorescein cleaved ERβ 258-503(G). In this experiment, we used only a 10th of the amount of ER: intein fusion protein used for the DTT elution in B).

Final remarks to specific aim 1:

Now, finally, we have a strategy that will allow us to obtain specifically labeled fluorescent ER. Unfortunately, right after we have worked out the purification strategy for these proteins my technician Lawrence Getubig left to attend art school in Boston and my postdoc Margarita Lib-Myagkov decided to attend medical school. I spent the last four months training new personnel and we haven't been able to perform the fluorescence spectroscopy analyses yet. Moerover, due to the takeover of Molecular Probes by Invitrogen, we have to find an alternative source to supply us with cysteine-fluorescein. Hence, I estimate that we will need another 6-9 months to finish this specific aim and to publish the results.

Last year John Katzenellenbogen (Tamrazi et al., 2003) published a fluorescence analysis of ERα showing that different ligands lead to changes in the fluorescence properties of the ER LBD. This study validates the rationale of our proposal. In their study, ERα was labeled via existing cysteine residues at positions 417 and 530 in the ligand binding domain, a strategy we initially considered but then dismissed because of the difficulty to control the specificity of the labeling process. Our studies on ERβ will complement their results and enable us to identify ERα- and ERβ-specific structural changes induced by SERMs.

Specific Aim 2

Analyze the role of the ER F-domain in the ligand-dependent relocation of H12

One goal of this grant proposal is the identification of receptor-specific structural elements that might participate in the ligand-dependent repositioning of H12 and in the recruitment of corepressors and coactivators. A prime candidate for such an activity is the F-domain, which extents the C-terminal end of H12. Despite mounting evidence for an important role of this domain in regulating ligand-dependent activities of ER, thus far most structural investigations have been performed with ER LBD deletion mutants that lack the F-domain. Since structural information for ER F-domains is not available yet, most of our structural considerations are based on the structure of the progesterone receptor (PR) F-domain (Williams and Sigler, 1998) (Fig. 6). To evaluate our conclusions, some of the following studies were performed with ER and PR in parallel.

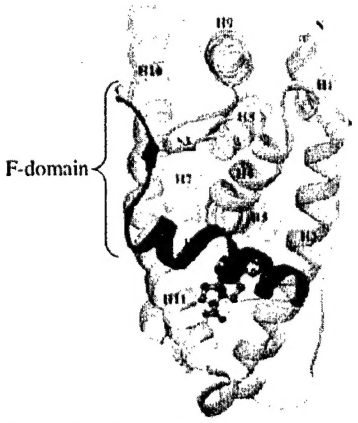


Fig. 6 Structure of the progesterone receptor (PR) ligand binding domain (Williams and Sigler, 1998)

Our experimental plan for this specific aim has been as follows:

a) Identification of the roles of ERα and ERβ F-domains
   - Cloning, expression and purification of wild ERα and ERβ LBD ± F-domain;
   - Characterization of the hormone binding ability of ERα and ERβ LBD ± F-domain;
   - Analysis of the contribution of the ER F-domains to coactivator and corepressor binding
   - Effect of the F-domain on the functional regulation of ER by coactivators

b) Preparation of PR and ER containing fluorescence-labeled F-domains
   - Identification of an experimental strategy to label the F-domain of PR
   - Cloning, expression and purification of fluorescence-labeled ER and PR F-domain variants
   - Hormone binding abilities of fluorescence-labeled ER and PR

c) Monitoring the movement of the F-domains of ER and PR in the absence and presence of various ligands

Aim 2a: Identification of the roles of ERα and ERβ F-domains

Cloning, expression and purification of wild ERα and ERβ LBD ± F-domain - We have cloned the ligand binding domains of human ERα and ERβ ± F-domain and expressed these proteins in the *E. coli* strain BL21DE3. At 37°C the ligand binding domains of ERβ and ERβ-F were partially soluble, whereas those of ERα and ERα-F were completely insoluble. However, expression at 13°C and coexpression of the chaperones GroEL/ES increased the solubility of these proteins and enabled us to obtain between 2 and 8 mg soluble protein per liter bacterial culture for all four ER constructs. ERβ LBD ± F-domain and ERα LBD ± F-domain were purified to homogeneity using affinity chromatography.

Characterization of the hormone binding ability of ERα and ERβ LBD ± F-domain - In *in vitro* hormone binding assays these purified proteins bind 17-β estradiol with the following dissociation constants: ERα/1.0 ± 0.5 nM, ERα-F/0.5 ± 0.2 nM, ERβ/1.4 ± 0.5 nM, and ERβ-F/1.2 ± 0.3 nM. Thus in case of ERβ, binding of estradiol is not influenced by the presence or absence of the F-domain, whereas removal of the ERα F-domain increased the affinity for 17-β estradiol by a factor 2. Both, ERα and ERβ bound the partial antagonist 4OH-tamoxifen with similar affinity (31.8 ± 8.1 nM and 30.1 ± 5.3 nM, respectively). Binding of 4OH-tamoxifen by ERβ was not influenced by the absence or presence of the F-domain, whereas the affinity of ERα for 4OH-tamoxifen decreased 5-fold in the absence of the F-domain. <u>These results demonstrate that the F-domain of ERα influences the affinity for ligands whereas the F-domain of ERβ has no affect on ligand binding.</u>

Analysis of the contribution of the ER F-domains to coactivator and corepressor binding -Transcriptional activity of ERα and ERβ depends on the interaction of these receptors with coactivators and corepressors. Most coactivators, such as the p160 coactivator GRIP1, have multiple nuclear receptor interaction sites, called NR-boxes, which bind to nuclear receptors with different affinities (Darimont et al., 1998). Using quantitative GST-pulldown experiments we determined that ERα displays the highest affinity for GRIP1 NR-boxes 2 and the lowest for NR-box 3, whereas ERβ does not discriminate between these NR-boxes (Fig. 7). Deletion of the F-domain of ERα increased binding to all NR-boxes while abolishing the selectivity for particular NR-boxes. Removal of the ERβ F-domain had no obvious consequence for the binding of GRIP1. <u>These results showed that the F-domain of ERα, but not that of ERβ, contributes to the affinity and selectivity of coactivators.</u>

Next we analyzed the ability of ERα±F to bind the corepressor NCoR in the presence of 4OH-Tam. Because tissues from mice that do not express NCoR are unable to inhibit the activity of ER in the presence of partial antagonists, the ability of a ligand to support NCoR binding appears to be directly linked to its function as a SERM (Jepsen et al. 2000). Similar to coactivators, the interaction of NCoR with nuclear receptors also depends on conserved amphipathic α-helices that interact with the hydrophobic groove in the receptor LBD (Hu and Lazar, 1999; Nagy et al., 1999; Perissi et al., 1999). To study the interaction of ER ± F-domain with NCoR, we have cloned and expressed two NCoR fragments containing these amphipathic motifs (2365-2239; 2453-2057) as fusions with an N-terminal glutathione-S-transferase (GST)-

tag and a C-terminal His_6-tag. After purification of these fusion proteins by Co^{2+}-affinity chromatography, we analyzed their binding to *in vitro* translated, S^{35}-labeled ERα and ERα-F in the presence of saturated levels of 4OH-Tam. In spite of many optimization attempts, binding of ERα and ERβ to these NCoR fragments was very weak (2-3 fold above background). Binding of NCoR to ERβ was not influenced by the F-domain, whereas binding of NCoR to ERα was consistently 3-fold higher in the absence than in the presence of the F-domain. Thus, the F-domain of ERα inhibits binding of both, coactivators and corepressors.

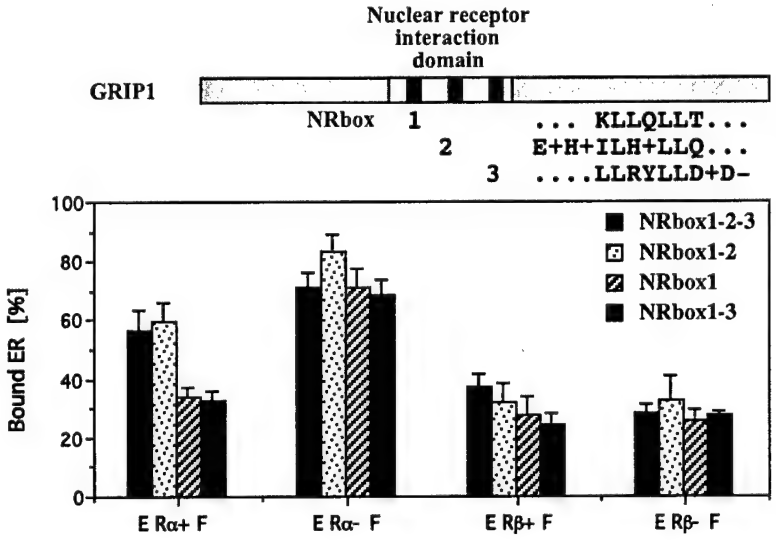


Fig. 7 Coactivator binding of ERs
The nuclear receptor interaction domain (NID) of p160 coactivator GRIP1 contains three "LxxLL" nuclear receptor-binding motifs (NR-boxes). To determine whether the F-domain contributes to coactivator binding and selectivity, we measured binding of ^{35}S-labeled ERα/β LBD ± F-domain to glutathione-S-transferase fusions of the GRIP1 NID. These experiments we performed with NID variants that contain either all three NR-boxes, NRbox 1 and 2, NRbox 1, or NRbox 1 and 3. NR-boxes were mutated by replacing the conserved LxxLL (L=leucine) motifs with AxxAA (A=alanine). The amount of bound receptor (relative to input) was quantified using a phosphorimager. The shown results are the average of more than three independent experiments.

Effect of the F-domain on the functional regulation of ER by coactivators - To determine whether the observed changes in coactivator binding lead to changes in the transcriptional activity of ERs, we monitored the transcriptional activity of ERα and ERβ ± F-domain using a reporter-based activity assay in transiently transfected CV1 cells. The presence or absence of the F-domain did not change the transcriptional activity of ERβ (data not shown), whereas in the absence of the F-domain the efficacy of the transcriptional activity of ERα increased 2-fold (Fig. 8A). Western blot analysis determined that the difference in the hormone responsiveness of ERα and ERα-F in CV1 cells is not caused by differences in the expression levels of these proteins.

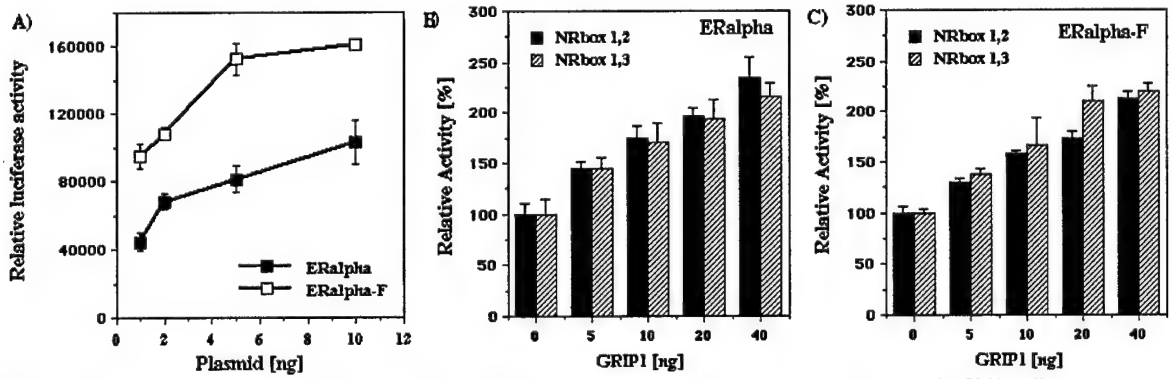


Fig. 8 A) Transcriptional activity of ERα and ERα-F monitored from an ERE-luciferase reporter in CV1 cells.
B and C) Response of the transcriptional activity of ERα and ERα-F in the presence of increasing concentrations of GRIP1 NRbox1,2 and GRIP1 NRbox 1,3. All assays have been performed in the presence of saturating concentrations of 17ß estradiol (10 nM).

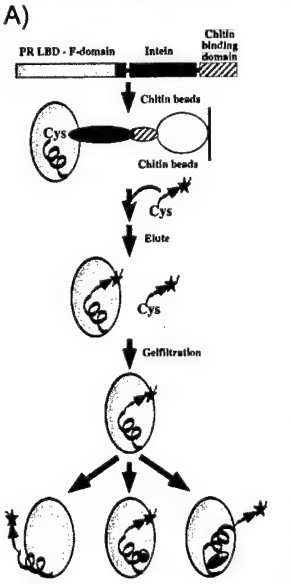
We next investigated whether the increased activity of ERα-F in CV1 cells might be caused by the increased affinity of this protein for NR-boxes 1 and 3 of GRIP1 (Fig. 7). Surprisingly, contrary to the increased affinity of ERα-F for the GRIP1 NR-box 1/3 in GST-pull down experiments, the absence or presence of the F-domain did not affect the response of ERα

to this GRIP1 variant (Fig. 8B, C). Thus, the increased transcriptional activity of ERα - F-domain in CV1 cells might not be caused by the observed increased affinity for certain GRIP1 NR-boxes.

Aim 2b: Preparation of PR and ER containing fluorescence-labeled F-domains

A possible explanation for the differences in the roles of the ERα and ERβ F-domains might be that the F-domain of ERα is linked back to the ER LBD core and controls the ligand-dependent localization of H12, whereas the F-domain of ERβ is a flexible, solvent exposed extension, which does not influence the movement of H12. Upon labeling the F-domains with a fluorophore, these structural differences could be monitored using time resolved fluorescence anisotropy.

Identification of an experimental strategy to label the F-domain of PR - To identify an efficient labeling strategy and to test whether structural changes in the F-domain could be detected by fluorescence spectropscopy, in a pilot study we used the progesterone receptor (PR), for which the structure and localization of the F-domain in the presence of an agonist has been determined by X-ray crystallography [Williams and Sigler, 1998]. In this structure the F-domain is linked to the LBD core via a short β-sheet. Hence, we expect the fluoro-phore to show only a slow rotational movement that corresponds to the rotation of the entire protein.



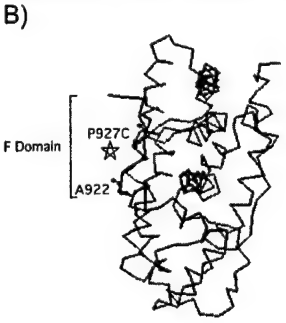


Fig. 9: A) Introduction of a fluorophore into the PR F-domain by intein-catalyzed protein splicing. The splicing event is mediated by a cysteine residue at the C-terminus of the PR LBD-F-domain, which also forms the linker between the PR LBD-F-domain and intein, and a cysteine residue at the N-terminus of the F-domain peptide.
B) Selection of the splice site and residue for the introduction of the fluorophore.

For the introduction of the fluorophore we chose a protein splicing approach as outlined in Fig. 9A. Based on the available structural information (Williams and Sigler, 1998), we decided to introduce the C-terminal cysteine residue at position A922 in the PR LBD and the fluorophore at position P927 (Fig. 9B). P927 follows the short β-strand in the PR LBD, which in the presence of agonists links the PR F-domain back to the LBD. Thus, the rotational freedom of a fluorophore introduced at this position should be constrained upon the formation of the β-sheet that links the F-domain to the LBD core. The corresponding PR LBD-intein gene was constructed using a PCR strategy and verified by DNA sequencing. Upon expression in *E. coli*, at room temperature about 90% of the expressed protein was found to be insoluble. Neither reducing the incubation temperature during expression nor the addition of progesterone increased the solublity of the PR LBD: intein fusion protein. However, the remaining 10% (about 0.5 mg/ liter culture) bound to chitin with high affinity. This simple affinity chromatography step yielded protein that is more than 90% pure. Incubation of 2 mg chitin-bound PR LBD: intein fusion protein with a 10-fold molar excess of F-domain peptide ((NH2-CGMVKPL LFHKK-COOH; synthesized by our Biotech core facility) resulted in 200 µg of more than 95% pure PR LBD-F-domain protein (Fig. 10) This was a very encouraging result. With the help of Molecular Probes (Eugene) we synthesized a F-domain peptide in which alanine 922 is exchanged by a fluorescein-labeled cysteine residue (C*). The fluorophore was introduced while the N-terminal cysteine was still protected. The resulting 12 amino acid long F-domain peptide (NH2-CGMVKC*LLFHKK-COOH) was purified by reverse phase chromatography.

Unfortunately, the yield of the labeled peptide was more than 10-fold less than that of the unlabeled F-domain peptide; moreover the labeled peptide was much less soluble. These two unfortunate circumstances reduced the yield of the splicing reaction, and in several attempts we have been unable to obtain more than 50 µg of labeled PR out of 2 mg chitin-bound PR LBD: intein fusion protein. To separate the unbound peptide from the labeled PR LBD, the chitin eluate needs to be fractionated by gelfiltration. This strategy requires that we concentrate the labeled protein. However, every concentration attempt failed because the protein appears to bind unspecifically to the concentrators. Thus, in order to make this approach workable we needed to find a way to increase the yield of coupling the fluorophore to the PR LBD.

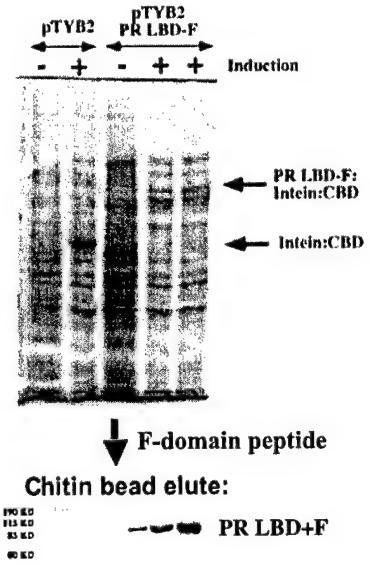


Fig. 10 Expression of PR LBD-F: intein (CBP) in *E. coli* BL21 DE3 and purification of PR LBD+F using protein splicing.

Cloning, expression and purification of ER and PR F-domain variants - Based on the direct labeling strategy by Kallenberger et al. (2003) using a fluorophore coupled cysteine, we (re)designed ERβ- and PR-intein fusion proteins to enable the direct labeling of their F-domains. In case of PR the F-domain was terminated at P927 followed by a cysteine residue that links this domain to the intein domain. Based on the available structural information, in the presence of an agonist, movement of the fluorophore should be restricted by the formation of the beta sheet between the F-domain and the LBD core (Fig. 6). Unfortunately for ER no structural information about the F-domain is available and the sequence similarity between the F-domains of ER and PR is too poor to make a prediction. As a start point we constructed four ER LBD: intein fusion proteins that give rise to the four fluorescence-labeled proteins ERαF1, ERαF2, ERβF1 and ERβF2 shown in figure 11.

```
ERα    DAHRLHAPTSRGGASVEE.TDQSHLATAGSTSSHSLQKYYITGEAEGFPATV
       .XX.X....X....X....xx.X.....XX....X.
ERβ    NAHVLRGCKSSITGSECSPAEDSKSKE.GSQNPQSQ

ERαF1 DAHRLHAPTSRGGASVEETDQSHLATAGSTSSHSLQPC*
ERαF2 DAHRLHAPTSRGGASVEETDQSHLATAPC*
ERβF1 NAHVLRGCKSSITGSECSPAEDSKSKEGSQNPQSQPC*
ERβF2 NAHVLRGCKSSITGSECSPAEDSKSKEPC*
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Fig. 11: Comparison of the F-domains of ERα and ERβ and the C-termini of ER LBD constructs containing labeled F-domains. "C*" denotes the residue that will be fluorescence-labeled.

Inclusion of F-domain sequences reduced the expression of the corresponding intein fusion proteins by a factor 2 but had no significant affect on the solubility of these proteins. In small scale experiments, the efficiency of binding of these proteins to chitin beads and their cleavage by either DTT or cysteine-fluorescein was similar as shown in figure 5.

Hormone binding abilities of fluorescence-labeled ER and PR - Due to our present shortage of cysteine-fluorescein, we only have very limiting quantities of the fluorescence labeled proteins. Therefore, we tested the hormone binding behavior of ER LBD F-domain constructs in which the intein domain has been cleaved by DTT. All four constructs bound estradiol with similar affinity than the corresponding wild type ER LBD + F-domain constructs.

Final remarks to specific aim 2:

This aim is in a similar state than specific aim 1: We have all the constructs to produce different fluorescence-labeled ER proteins, and we have a working strategy and trained personnel to purify and label these proteins. Now we have to find a new source to supply us with cysteine-fluorescein, purify larger quantities of these proteins and perform the fluorescence

spectroscopy analyses. These studies will be performed together with the remaining experiments of specific aim 1. Hence, we estimate that we will need another 6-9 months to finish these studies and to publish the results. These studies would provide the first insights into ligand-dependent structural changes involving the F-domain.

A note about the personnel situation

We were prepared that this project is technically difficult and will require experimental adjustments. However, what hampered our progress most was the personnel situation. My technician Galina Kouzmischeva and my postdoc Christian Pullen, who were they driving forces of this project, had to leave during the first term of this grant due to visa reasons. Their replacement Lawrence Getubig and Margarita Lib-Myakgov had very little experience with molecular biology and protein purification and needed extensive training. Just when they began to work independently, Lawrence accepted an offer to attend an art school in Boston and Margarita decided to go back to medical school. Josh Goodley, a graduate student who was interested in this project decided after his comprehensive exam to move to a less biophysical field and finally left our graduate program to work in a company in Los Angeles. My undergraduate student Kori Beyer, who participated in these studies experienced severe health problems, which forced her to drastically reduce the time she spent on doing research. Thus, during the last few months this project was continued to a large extent by myself and several rotation students. Presently, I am training my new technician Leah Ulsted, who will assist me in finishing the remaining experiments.

Key Research Accomplishments

Aim 1. Monitor the location and dynamic of the ER H12 in the absence and presence of various ligands and develop a high throughput screen for the identification of new potential SERMs

Our results in aim 2 indicate that the structural analysis of ERβ is likely easier than that of ERα (higher solubility, no contribution of the F-domain to ligand- and cofactor-binding). Hence, our focus has been on the identification of a strategy to label the ERβ LBD ± F-domain.

- Based on available structural information, we selected the following sites for the introduction of the fluorophores: Potential donor sites - N496, A497, H498; potential acceptor sites - L477 (A1-Agonist monitor), T323 (A2-Antagonist monitor). To determine whether labeling of these residues with fluorophores would interfere with ER folding, stability, and ligand binding, we replaced these residues by tryptophan, purified the corresponding proteins and measured the affinity of these proteins for 17ß-estradiol and 4OH-tamoxifen. Replacement of these residues by tryptophan had no or only minor effects on hormone binding.
- We constructed ER expression constructs containing nonsense-codons at the sites selected above as well as corresponding suppressor tRNAs that could be used to introduce fluorescence-labeled amino acids into ER. We tested several *in vitro* expression systems and identified that rabbit reticulocyte lysate system gives the highest yield of hormone-binding competent ER. We made several attempts to express fluorescence-labeld ER using this system. However, in each attempt the yield of labeled proteins was too low for our purpose.
- We tested protein splicing as an alternative approach to introduce fluorecence labels into ER. For this purpose we designed, expressed and purified two sets of ERβ LBD: intein fusion proteins: The first set of proteins enables the ligation of the ERβ LBD core with *in vitro* synthesized, fluorescence-labeled H12; the second set allows the attachement of cysteine-fluorescein at the C-terminus of H12 of ERβ. Fluorescence-labeled H12 peptides revealed to be too insoluble to efficiently catalayze the splicing reaction. However, the incorporation of cysteine-fluorescein works well and will enable us to obtain specifically labeled ER LBDs. The only remaining obstacle is that we have to identify a source to obtain larger quantities of cysteine-fluorescein.

Aim 2. Analyze the role of the ER F-domain in the ligand-dependent relocation of H12

- We constructed, expressed and purified ERα and ERβ ligand binding domains that either contain or lack the F-domain.
- Hormone-binding studies revealed that the F-domain of ERβ has no effect on the binding of 17ß-estradiol or 4OH-tamoxifen. However removal of the F-domain of ERα increased the affinity of ERα LBD for 17ß-estradiol 2-fold, and decreased the affinity for 4OH-tamoxifen 5-fold. These results demonstrate that the F-domain of ERα modulates ligand binding in a ligand-dependent manner.
- *In vitro* binding studies showed that the F-domains of ERα and ERβ have different affects on the binding of coactivators and corepressors. Removal of the F-domain or ERα increased the affinity and changed the specificity of ERα for corepressors and coactivators, whereas the removal of the F-domain of ERβ had no detectable effect.
- In reporter assays using transiently transfected CV1 cells, removal of the F-domain resulted in a 2-fold increase in the transcriptional activity of ERα. This activity difference is not caused by differences in the expression levels of ERα and ERα-F. Contrary to its affect on binding GRIP1 NR-boxes *in vitro*, in CV1 cells the absence or presence of the F-domain did not affect the response of ERα to GRIP1. Thus, the increased transcriptional activity of ERα-F in CV1 cells might be caused by factors other than the p160 coactivator GRIP1.
- Since contrary to the F-domains of ERα and ERβ the structure of the F-domain of the progesterone receptor (PR) has been solved, in a pilot experiment we attempted to introduce fluorescence-labels into the PR F-domain by linking *in vitro* synthesized, labeld F-domains to the PR LBD via protein splicing. For this purpose, we constructed, expressed and purified PR LBD: intein fusion proteins. Moreover, we synthesized and purified a 12 amino acid long PR F-domain peptide that contains a fluorescein-coupled cysteine residue instead of PR residue A922. While the PR LBD domain was efficiently released from the intein domain in the presence of DTT, the fluorescence-labeled peptide was too insoluble to catalyze the splicing reaction efficiently.
- With respect to a recently published study by Kallenberger et al. (2003), we (re)designed, expressed and purified PR LBD: intein and ER LBD: intein fusion proteins in which the F-domain can be labeled through C-terminal coupling of cysteine-fluorescein. This strategy avoids the difficulties caused by the low yield and solubility of the fluorescent labeled H12 peptide. In test trials these proteins were readily released from intein by DTT and could be specifically labeled by cysteine-fluorescein. Hormone-binding studies revealed the competency of these proteins to bind 17ß-estradiol.

Reportable Outcomes

Publications:

Due to the delay in finding efficient labeling strategies and the mentioned personnel situation, this work has not been finished and published yet. After completion of the fluorescence measurements, we plan to publish these studies in two reports:

1. Fluorescence-dependent analysis of ligand-dependent structural changes in ERβ
   C. Pullen, C. Beyer, B. Darimont
2. The F-domains of ERα and ERβ play different roles in regulating binding of ligands and coregulators
   C. Pullen, J. Goodley, C. Beyer, B. Darimont

Abstracts/Presentation:

- C. Pullen, J. Goodley, B. Darimont
  Novel strategies for the identification and characterization of selective estrogen receptor modulators
  Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Orange County Convention Center, Orlando, Florida, September 25-28, 2002

- C. Pullen, J. Goodley, B. Darimont
  Novel strategies for the identification and characterization of selective estrogen receptor modulators
  Annual Retreat of the Institute of Molecular Biology, University of Oregon, Silverfalls, Oregon, September 22/23, 2002
- C. Pullen, J. Goodley, M. Lib-Myagkov, B. Darimont
  Novel strategies for the identification and characterization of selective estrogen receptor modulators
  Annual Retreat of the Institute of Molecular Biology, University of Oregon, Silverfalls, Oregon, September 23/24, 2003

Employment:
In Fall 2002, Christian Pullen accepted a scientist position at a pharmaceutical company in Germany.

Experience/Training:
Undergraduate research experience: Corinna Beyer
Research Associate research experience: Galina Kouzmitscheva, Lawrence Getubig
Postdoctoral training: Christian Pullen, Margarita Lib-Myagkov
Doctoral training: Joshua Goodley

Conclusions

While the characterization of ligand-bound estrogen receptor ligand binding domains (ER LBDs) by X-ray crystallography gave many interesting insights into the mechanisms of ligand binding and ligand interpretation, these structures do not provide a coherent explanation for the tissue- and receptor isotype-specific activity displayed by many ligands. Our results indicate that the F-domain, a C-terminal extension of the ligand binding domain, which is not included in the known structures of ER LBDs, regulates hormone and coregulator binding in a receptor-specific manner. Moreover, receptors bound to SERMs, which display both agonistic and antagonistic activities, might differ from receptors bound to pure agonists or antagonists in terms of the dynamics rather than the nature of the ligand-induced structural changes. To monitor the dynamics of structural changes in the ER LBD upon ligand binding, we have constructed a series of ER LBD derivatives containing fluorescence-labeled residues. Fluorescence analyses of these proteins will not only give novel insights into the actions of SERMs but also provide a powerful strategy for the identification of novel SERMs that might improve the treatment of breast cancer.

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Appendices

None